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## Exercise training, Beta-blocker treatment and Muscle function in Pulmonary Arterial Hypertension

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2010

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### **citation for published version (APA)**

de Man, F. S. (2010). *Exercise training, Beta-blocker treatment and Muscle function in Pulmonary Arterial Hypertension*. [PhD-Thesis - Research and graduation internal, Vrije Universiteit Amsterdam].

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# 1

## **General Introduction and Outline of the Thesis Pulmonary Arterial Hypertension**



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## Pulmonary Arterial Hypertension

Pulmonary Arterial Hypertension (PH) belongs to the first class of clinical pulmonary hypertension (table 1),<sup>1</sup> and is defined as a mean pulmonary artery pressure (mPAP)  $\geq 25$  mmHg at rest with a pulmonary arterial wedge pressure  $\leq 15$  mmHg, as assessed by right heart catheterization.<sup>2</sup> PH is a rare disease with a prevalence of 15/million patients.<sup>3</sup> The majority of patients is female (male/female ratio of 1:2) with variable age (peak-prevalence at age of 50 years).<sup>3</sup>

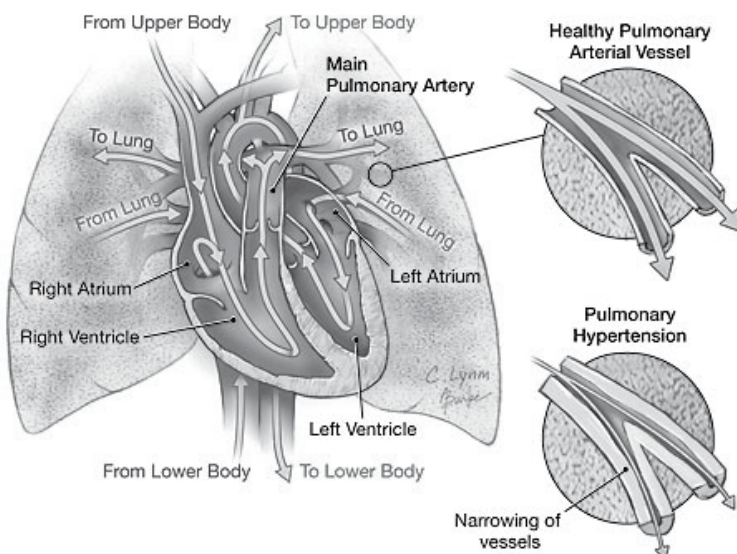
**Table 1.1** Clinical classification of Pulmonary Hypertension (Dana Point 2008)

1. Pulmonary Arterial Hypertension
2. Pulmonary Hypertension owing to left heart disease
3. Pulmonary Hypertension owing to lung diseases and/or hypoxia
4. Chronic Thromboembolic Pulmonary Hypertension
5. Pulmonary Hypertension with unclear multifactorial mechanisms

PH is characterized by excessive pulmonary vascular remodelling, resulting in high pulmonary artery pressures (Figure 1). Eventually, the right ventricle cannot adapt to the increase in afterload, and PH-patients die as a consequence of overt right heart failure (RHF). Even under maximal treatment, the prognosis of PH-patients remains grim, with a 5-year survival of about 50%.<sup>4</sup> The main symptoms of the patients are reduced exercise tolerance, shortness of breath

**Figure 1.1** Pathogenesis of Pulmonary Arterial Hypertension

Pulmonary Arterial Hypertension is characterized by excessive pulmonary vascular remodelling, resulting in high pulmonary vascular resistance. As a consequence, right ventricular afterload increases as the right ventricle has to pump blood into the lungs against a higher resistance. Eventually, the right ventricle cannot adapt to the increase in afterload, and PH-patients die as a consequence of overt right heart failure.



and symptoms related to RHF such as edema. In the absence of a cure, a better understanding of the etiology of these symptoms, might provide new additional therapeutic strategies to improve prognosis and quality of life of PH-patients: the primary aim of this thesis.

### Reduced exercise capacity

The main physiological basis of reduced exercise capacity in PH is a limitation of (the increase in) cardiac output.<sup>5-7</sup> It is well known from left heart failure patients, who are also characterized by a low cardiac output state,<sup>8</sup> that exercise capacity can be improved by exercise training.<sup>9</sup> Moreover, it was even reported that exercise training could delay the progression of heart failure and improve prognosis in the most severe patients.<sup>10</sup> For this reason there is a rationale to investigate if exercise training might be also beneficial in PH.

However, in contrast with most left heart failure patients, right heart failure in PH is afterload related. Augmentation of cardiac output during exercise training will even further increase afterload,<sup>11</sup> which could lead to sudden cardiac failure. Therefore, exercise training in PH is, at present, contra-indicated, although this advice is hardly supported by data. For this reason we investigated in **Chapter 2** the effects of exercise training in a rat model for PH with two different phenotypes: stable PH with preserved cardiac function and progressive PH with right heart failure. Encouraged by the findings that exercise training in these rats was indeed beneficial in stable PH, we investigated in **Chapter 3** the effects of a 12 week training program in stable patients with idiopathic PH. As previous findings in left heart failure indicated that the beneficial effects of exercise training on exercise capacity could mainly be ascribed to improvements in skeletal (quadriceps) muscle function and muscle efficiency,<sup>12,13</sup> we assessed the effects of exercise training on exercise capacity, together with quadriceps muscle function and morphology.

### Right heart failure

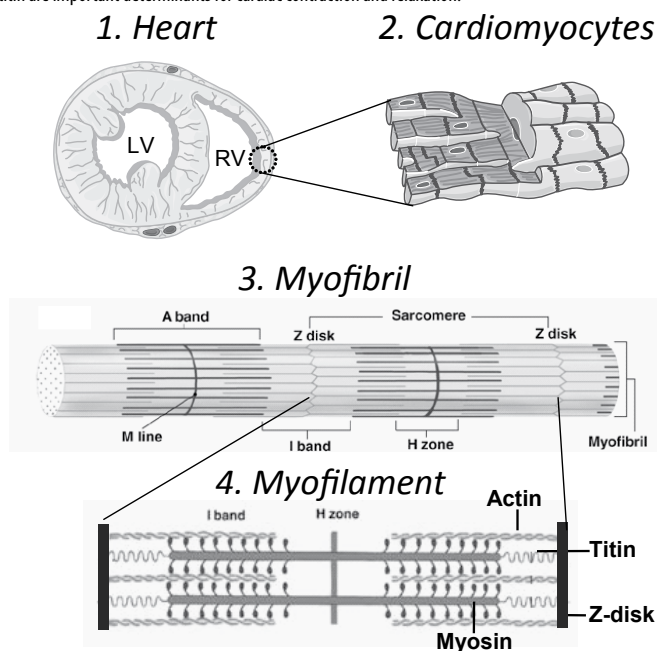
Several longitudinal clinical studies have revealed the importance of indices of right ventricular function on long-term prognosis of patients with PH.<sup>14-16</sup> Insight in the pathophysiological mechanism of right heart failure is therefore necessary to develop new therapeutic options directly aiming to improve RV function.

In left heart failure, it has been described that chronic elevated levels of catecholamines have detrimental effects on cardiac function by its direct cardiotoxic effects and via reduced  $\beta$ -adrenergic receptor signaling.<sup>17,18</sup> The  $\beta$ -adrenergic receptor pathway is the upstream activator of myofilament phosphorylation regulating cardiac contractility and relaxation (See Figure 2 for the localization of the myofilament within the heart).<sup>19-21</sup>

Also in right heart failure reduced  $\beta$ -adrenergic receptor density has been reported,<sup>22</sup> but the functional consequences remain to be elucidated. Therefore, we investigated in **Chapter 4** if altered myofilament phosphorylation and function could in part underlie right heart failure in progressive PH in comparison with stable PH.

**Figure 1.2** Schematic representation of myofilament localisation within the right ventricle

1) The heart (left ventricle, septum, right ventricle) is composed of cardiomyocytes (2). Myofibrils are located within the cardiomyocytes consisting of serially linked sarcomeres that lie between the two successive Z-disks. 3) The myofilament proteins actin (thin filament), myosin (thick filament) and titin are important determinants for cardiac contraction and relaxation.



Furthermore,  $\beta$ -blocker therapy is a standard therapeutic strategy in the treatment of patients with left heart failure to reduce the detrimental effects of chronic elevation of catecholamine levels. Notwithstanding several indications of sympathetic overactivity in PH,<sup>23,24</sup>  $\beta$ -blockers are contra-indicated for the treatment of PH,<sup>25</sup> as it is thought that PH patients do not tolerate its (transient) myocardial depressant effects. This recommendation is partially substantiated by the findings of previous studies demonstrating that acute administration of  $\beta$ -blocker led to arterial-ventricular uncoupling and  $\beta$ -blocker withdrawal improved exercise capacity.<sup>26,27</sup> However, these studies only described acute effects of  $\beta$ -blockers and used earlier generations and non-selective  $\beta$ -blockers. We therefore investigated in **Chapter 5** the effects of chronic cardiac-specific  $\beta$ -blocker treatment in progressive PH on mortality and right ventricular function, and specifically assessed arterial-ventricular coupling by pressure-volume relations.

#### Sensation of dyspnoea

Dyspnoea is defined as an uncomfortable sensation of breathing.<sup>28</sup> Approximately 87% of PH-patients suffer of the symptom dyspnea.<sup>5</sup> Already in 1976, it has been demonstrated that the sensation of dyspnoea is related to the required inspiratory muscle strength to produce

inspiratory flow.<sup>29</sup> Hence, the sensation of dyspnoea increases when respiratory muscles are unable to generate sufficient force.

Two recent reports have revealed that patients with PH are not able to increase maximal inspiratory pressures.<sup>30,31</sup> This implies that reduced respiratory muscle function might play a role in the sensation of dyspnoea in PH-patients. It should be noted, however, that the capacity to generate negative intrathoracic or transdiaphragmatic pressure is an indirect measure of inspiratory muscle strength. These methods do not solely measure muscle function, but central drive, nerve function and neuromuscular transmission as well, so that determination of inspiratory muscle strength per se is not possible *in vivo*.

The diaphragm muscle is the main muscle of inspiration. Although studying diaphragm muscle contractile properties will give essential information on the relation between inspiratory muscle weakness and dyspnoea in patients with PH, obtaining human diaphragm biopsies can not be carried out for ethical reasons. We therefore investigated in **Chapter 6** the contractile properties and morphology of the diaphragm muscle fibers in a rat model for PH. Interestingly, we could also assess diaphragm muscle morphology of two patients who died of PH.

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# 2

## Opposite Effects of Training in Rats with Stable and Progressive Pulmonary Hypertension



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*Circulation* 2009; 120: 42-49

## ABSTRACT

*Background* - Exercise training in pulmonary arterial hypertension (PH) is a promising adjunct to medical treatment. However, it is still unclear whether training is beneficial for all PH-patients. We hypothesized that right ventricular adaptation plays a pivotal role in the response to training.

*Methods and Results* – Two different dosages of monocrotaline were used in rats to model stable PH with preserved cardiac output, and progressive PH developing right heart failure. Two weeks after injection, PH was confirmed by echocardiography, and treadmill training was initiated. Rats were trained for four weeks, unless manifest right heart failure developed earlier. At end of study protocol, all rats were functionally assessed by endurance testing, echocardiography and invasive pressure measurements. Lungs and hearts were further analyzed for quantitative histomorphologic analyses.

In stable PH, exercise training was well tolerated and markedly increased exercise endurance (from  $25 \pm 3.9$  to  $62 \pm 3.9$  min;  $p < 0.001$ ). Moreover, capillary density increased significantly (from  $1.21 \pm 0.12$  to  $1.51 \pm 0.07$  capillaries per cardiomyocyte;  $p < 0.05$ ). However, in progressive PH exercise training worsened survival (hazard ratio 2.7, 95%CI: 1.1-14.2) and increased pulmonary vascular remodeling. In addition, training induced widespread leukocyte infiltration into the right ventricle (from  $135 \pm 14$  to  $276 \pm 18$  leukocytes per  $\text{mm}^2$ ;  $p < 0.001$ ).

*Conclusions* – In our rat model, exercise training was found to be beneficial in stable PH, but detrimental in progressive PH. Future studies are necessary to address the clinical implications of our findings.

## INTRODUCTION

Pulmonary arterial hypertension (PH) is characterized by progressive pulmonary vascular remodeling, which importantly increases right ventricular (RV) afterload, eventually leading to right heart failure and premature death.<sup>1</sup> Traditionally, PH-patients were advised to limit physical activity, because of risk of fatal cardiovascular compromise.<sup>2</sup>

Recent developments have however challenged this view.<sup>3</sup> Firstly, prognosis of PH-patients improved by introduction of various potent PH-specific medications in the last decades.<sup>4</sup> Secondly, several studies have demonstrated beneficial effects of training in patients with chronic obstructive pulmonary disease and with congestive heart failure, and training was beneficial even for the most severely affected patients (GOLD IV, NYHA IV) often suffering from secondary pulmonary hypertension.<sup>5,6</sup> Finally, in a recent clinical trial with thirty *stable* PH-patients under optimized medical treatment, Mereles *et al.* reported marked improvement in exercise capacity and quality of life after exercise training.<sup>7</sup>

Although training might be a promising adjunct to medical treatment in PH, it remains to be elucidated whether exercise training is beneficial for *all* PH-patients, and what its effect is on RV function and remodeling. RV adaptation might be a discriminating factor for responsiveness to training. During exercise, pulmonary artery pressures and RV afterload increases,<sup>8</sup> resulting in a transient elevation of RV wall stress. Although this is unknown for right heart failure, for left heart failure it has been demonstrated that even a temporary elevation of wall stress can up-regulate local pro-inflammatory factors, leading to leukocyte infiltration into the myocardium.<sup>9</sup> We hypothesized that such a pro-inflammatory reaction in the right ventricle might outbalance the positive effects of exercise training, especially in the presence of RV maladaptation to pressure overload.

We therefore conducted an experimental study and assessed the effects of exercise training in two phenotypes of PH; namely stable PH with a preserved cardiac output at rest, and progressive PH developing right heart failure. Using a comprehensive set of physiologic and pathologic endpoints, we documented beneficial effects in stable PH, but detrimental effects in progressive PH.

## METHODS

All experiments were approved by the Institutional Animal Care and Use Committee at the VU University.

Experimental pulmonary hypertension

Male Wistar rats were used (56 in total, 150-175g; Harlan, Horst, the Netherlands). PH was induced by a single subcutaneous injection of monocrotaline (MCT; Sigma-Aldrich, Zwijndrecht, The Netherlands) dissolved in sterile saline.

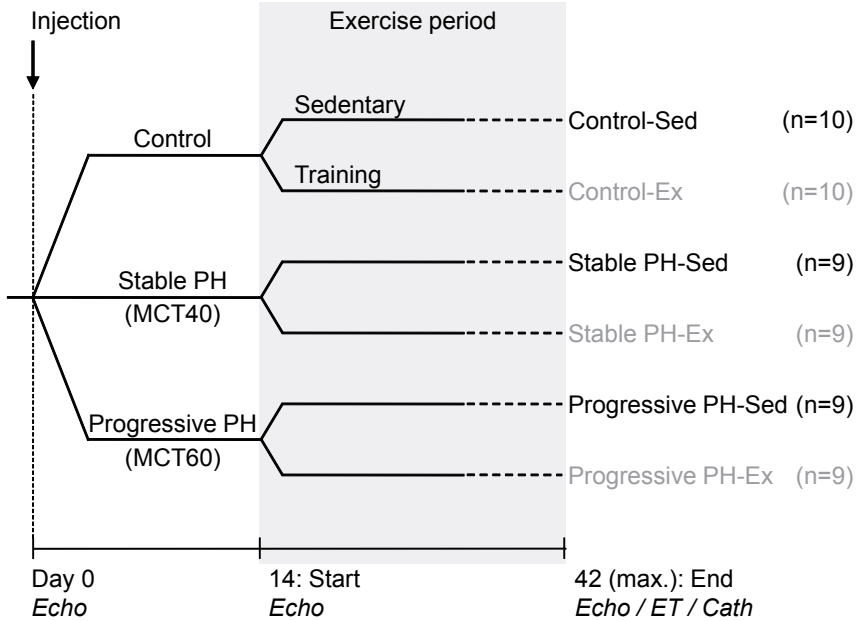
MCT 60 mg/kg body mass was used to model progressive PH developing right heart failure (n = 18); with a dose of 40 mg/kg MCT, stable PH with a preserved cardiac output was mimicked (n = 18).<sup>10;11</sup> The control group was injected with saline only (n = 20).

Study design and training program

The exercise program was adopted from a validated exercise program for Wistar rats, described by Fenning and Harrison, *et al.*<sup>12</sup>

In the first week, all rats were accustomed to treadmill running: mild electrical stimulation was used to encourage the rats to run. Then, rats were randomly assigned to any of the three experimental groups (Control, Stable or Progressive PH) and injected accordingly with MCT or saline (Figure 1). In the following two weeks, all rats were placed on the treadmill for one minute a day (five times a week, at a constant speed of 13.3 m/min, no slope). After these two weeks, the rats were again randomly assigned to an exercise training program (control-Ex, stable PH-Ex, progressive PH-Ex; 5x /week; 30 min; 13.3m/min; no slope) or the sedentary

**Figure 2.1** Study design  
The effect of exercise training was studied in two distinct phenotypes of established pulmonary hypertension (stable and progressive PH).  
Abbreviations: Start = start of exercise period (14 days after injection); End = end of study protocol (when manifest signs of right heart failure developed, or 42 days after MCT-injection). Echo = echocardiographic evaluation; ET = endurance testing; Cath = RV catheterization.



group (control-Sed, stable PH-Sed, progressive PH-Sed; 5x /week; 1 min; 13.3m/min; no slope). The level of training represented moderate exercise intensity ( $\approx 50\% \text{VO}_2\text{max}$ ).<sup>13</sup> Animals were trained for maximally four weeks (from day 14 until day 42 after MCT-injection). Rats that developed manifest clinical signs of right heart failure (defined as:  $>5\%$  loss of body mass/day and/or respiratory distress, cyanosis, lethargy) were euthanized early, in keeping with the protocol approved by the institutional animal care committee. Manifest right heart failure was the survival endpoint and recorded as an event in the survival analysis.<sup>14</sup>

#### Endurance test

Only on rats that completed the four-week exercise program, endurance testing was performed.<sup>12</sup> The treadmill was set at a constant speed of 15 m/min, and a slope of 20 degrees. The time from start-until-exhaustion was used as a measure for exercise endurance of the rats. Exhaustion was established when the rats accepted the electric stimulus three consecutive times as opposed to running. The maximal running time was 90 minutes, which was achieved by all control rats.

#### Hemodynamic evaluation

##### *Echocardiography:*

The rats were evaluated by echocardiography at baseline (just before they received their injection), at start of training, and at the end of the study protocol (when manifest signs of right heart failure developed, or 42 days after MCT-injection). Transthoracic echocardiographic measurements (ProSound SSD-4000 system equipped with a 13 MHz linear transducer (UST-5542), Aloka, Tokyo, Japan) were performed on anaesthetized but spontaneously breathing rats (isoflurane 2.0% in 1:1 O<sub>2</sub>/air mix; Pharmachemie, Haarlem, The Netherlands), as described before.<sup>15</sup>

Analyses were performed off-line (Image-Arena 2.9.1, TomTec Imaging Systems, Unterschleissheim / Munich, Germany). Measured parameters for cardiac and right ventricular function were: Doppler-derived stroke volume, cardiac output, and tricuspid annular plane systolic excursion (TAPSE). Parameters for RV remodeling were: RV end diastolic diameter (RVEDD) and RV wall thickness. Parameters for pulmonary vascular remodeling were: pulmonary artery acceleration time normalized for cycle length (PAAT/cl) and pulmonary vascular resistance (PVR).

Disease progression of PH during the period of exercise training was expressed as percentage change in hemodynamics over time, i.e. change in cardiac output (CO):

$$[\Delta\text{CO}] = \frac{[\text{CO}_{\text{END OF PROTOCOL}}] - [\text{CO}_{\text{START OF TRAINING}}]}{[\text{CO}_{\text{START OF TRAINING}}]} * 100\% / [\text{days of training}].$$

Other parameters for disease progression ( $\Delta\text{SV}$ ,  $\Delta\text{TAPSE}$ , and so on) were calculated similarly.

*Non-invasive estimation of RV systolic pressures, pulmonary vascular resistance, and RV wall stress:*

The relationship between PAAT/cl and RV systolic pressure (RVSP), measured at the end of the study protocol, were used to non-invasively estimate RVSP (eRVSP) at baseline and at the start of training:<sup>51,52</sup>

$$[eRVSP] \approx 142 * e^{(-11*[PAAT/cl])}.$$

Pulmonary vascular resistance (PVR) was estimated by Poiseuille's law:<sup>53-56</sup>

$$[PVR] = \frac{[mean PAP] - [PCWP]}{[cardiac output]} \approx \frac{(0.61 * [RVSP] + 2mmHg)}{[cardiac output]}.$$

RV wall stress was estimated using LaPlace's law:<sup>55</sup>

$$[RV wall stress] = \frac{[RVSP] * [RVEDD]}{4 * [RV wall thickness]}.$$

*Invasive RV-pressure measurements:*

At the end of the study protocol, open-chest RV catheterization was performed under general anesthesia in all animals (isoflurane 2.0% in 1:1 O<sub>2</sub>/air mix), as described before.<sup>16</sup> Before the procedure, the rats were intubated (16 G Teflon tube) and attached to a mechanical ventilator (Micro-Ventilator, UNO, Zevenaar, The Netherlands; ventilator settings: breathing frequency 80/min, pressures 9/0 cmH<sub>2</sub>O, inspiratory/expiratory ratio 1:1). The right ventricle was approached via a lateral right thoracotomy through the fifth intercostal space. RV pressures were recorded by the use of a high-fidelity catheter-tip transducer (Mikro-Tip SPR-671, Millar Instruments, Houston TX). Analyses were performed when steady state was reached over an interval of at least 10 s and averaged.

*Histology*

After the final hemodynamic assessment, the rats were euthanized by exsanguination (under isoflurane), and heart, lungs and other major organs were harvested. Lungs were weighed, the airways of the left lobe subsequently filled with a 1:1 mix of saline and cryofixative (Tissue-Tek O.C.T. compound, Sakura Fintek Europe, Zoeterwolde, The Netherlands), and snapfrozen in liquid nitrogen. The right lobe was used to measure the dry/wet lung mass ratio. The heart was perfused, weighed, dissected and snapfrozen in liquid nitrogen.

*Histomorphometric analysis:*

*Histomorphomogy of the lungs*

Pulmonary sections (5 µm) were stained with Haematoxylin & eosin and Elastica von Giesson for morphometric analysis of vascular dimensions, as described before<sup>59,510</sup>. Minimally fifty

transversally cut pulmonary arterioles, randomly distributed over the lungs with an outer diameter between 25 and 100  $\mu\text{m}$ , were measured, using ImageJ. Relative wall thickness of pulmonary arterioles was calculated as<sup>59</sup>:

$$[\text{PA wall thickness}] = \frac{2 * [\text{medial wall diameter}]}{[\text{external diameter}]} * 100\%$$

### *Histomorphomogy of the heart*

#### *Cardiomyocyte cross sectional area:*

Cardiac cryosections (5  $\mu\text{m}$ ) were stained with Haematoxylin & eosin to determine LV and RV cardiomyocyte cross sectional area (CSA).<sup>57</sup> ImageJ was used for image analysis (ImageJ for Windows 1.39a, National Institutes of Health, Bethesda MD), taking the pixel-to-aspect ratio into account. Cardiomyocyte size for each ventricle was expressed as the average CSA of minimally twenty transversally cut cardiomyocytes at the level of the nucleus, randomly distributed over the ventricles.

#### *Cardiac fibrosis:*

Picrosirius red staining was used for analysis of cardiac fibrosis. By means of an internally validated ImageJ-macro, cardiac fibrosis was automatically detected.<sup>58</sup> LV and RV fibrosis were expressed as the percentage tissue area positive for collagen, measured over minimally three randomly chosen areas per ventricle.

#### *Cardiac inflammation and cappilarization:*

Analysis of capillary density and cardiac inflammation was performed by using quantitative immunofluorescence microscopy. Briefly, cardiac cryosections (5  $\mu\text{m}$ ) were incubated for 60 min with primary CD31- (1:35; sc-1506-R, Santa Cruz Biotechnology, Santa Cruz CA) and CD45-antibodies (1:25; sc-53045, Santa Cruz) for capillary density and leukocyte infiltrations, respectively, followed by appropriate secondary antibody staining as well as WGA (glycocalyx) and DAPI (nuclei) counterstaining. Image acquisition was performed on a Marianas digital imaging microscopy workstation (Intelligent Imaging Innovations (3i), Denver CO). SlideBook imaging analysis software (SlideBook 4.2, 3i) was used to semi-automatically quantify the images. Capillary density was expressed as the number of capillaries per cardiomyocyte or number of capillaries per section area, measured in at least three randomly chosen areas per ventricle, where cardiomyocytes were transversally sectioned.<sup>17</sup> Leukocyte infiltration was expressed as the number of positive CD45-nuclei per section area, measured over minimally three randomly chosen areas per ventricle.<sup>18</sup>



### Statistical analysis

All analyses were performed in a blinded fashion. All data were verified for normal distribution. Data are presented as mean±SEM and analyses were performed on all rats, unless stated otherwise. A p-value < 0.05 was considered significant.

Survival estimates were performed by Kaplan Meier–analyses, with post-hoc comparisons performed by log-rank test. Hazard ratios were calculated by the proportional hazards model. For all other *in vivo* data, two-way analysis of variance was used; Interaction between PH-status and training-status was tested, and subsequently Bonferroni post-hoc tests were performed (training vs. sedentary in the three experimental groups). All reported p-values of post-hoc comparisons are Bonferroni corrected (SPSS 16.0 for Windows, SPSS, Chicago IL).

For the histological data, multilevel analysis was used to correct for the non-independence of successive measurements of cross sectional areas and PA wall thickness per animal (MLwiN 2.02.03, Center for Multilevel Modelling, Bristol, UK).<sup>19</sup>

The authors had full access to the data and take responsibility for its integrity. All authors have read and agree to the manuscript as written.

## RESULTS

### Established pulmonary hypertension at start of training

Estimated RVSP (using PAAT/cl) at start of training was elevated for stable PH and progressive PH, compared to control (eRVSP, stable PH: 36±2.8 mmHg, progressive PH: 48±3.5 mmHg, control: 26±2.3 mmHg, p<0.001; Table S-1). In addition, compared to control, PVR for stable

**Table S2-1** Echocardiographic data at start of training

	Control (n=20)	Stable PH (n=18)	Progressive PH (n=18)
<b>Echocardiography</b>			
Cardiac output (mL/min)	119±4	117±3	127±5
Stroke volume (mL)	0.29±0.01	0.30±0.06	0.32±0.01
Heart rate (bpm)	405±5	394±5	399±5
TAPSE (mm)	3.6±0.1	3.5±0.1	3.3±0.1
RV wall thickness (mm)	0.96±0.01	1.08±0.02 ###	1.07±0.03 ##
RVEDD (mm)	3.6±0.1	3.6±0.1	3.6±0.1
PAAT/cl (°100)	17±1	13±1 #	11±1 #### †
eRVSP (mmHg)	26±2	35±3 #	48±4 #### †
PVR (mmHg/mL/min)	0.14±0.01	0.20±0.01 #	0.23±0.02 #

Echocardiographic characteristics control vs. stable PH vs. progressive PH at the start of training confirmed the pulmonary hypertensive status of MCT-treated rats. A strong MCT-dose dependent response was seen for PAAT/cl, eRVSP, PVR and RV wall thickness (p<0.001).

All data are presented as mean±SEM. #: p<0.05; ##: p<0.01; ###: p<0.001 vs. control; †: p<0.05 vs. stable PH.

Abbreviations: TAPSE = tricuspid annular plane systolic excursion; RVEDD = RV end diastolic diameter; PAAT/cl = normalized pulmonary artery acceleration time; eRVSP = estimated RV systolic pressure; PVR = pulmonary vascular resistance.

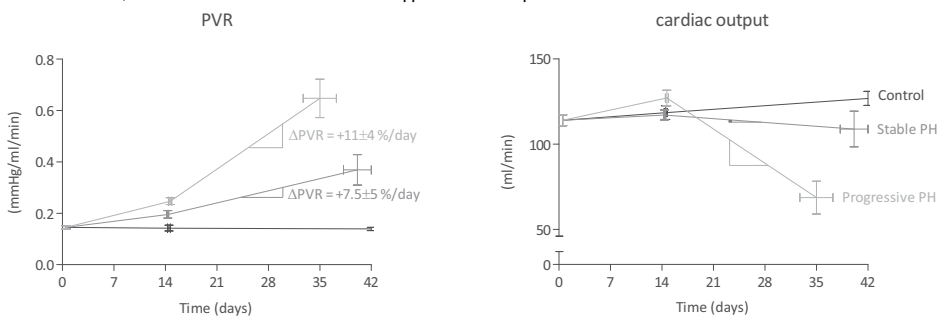
and progressive PH was higher as well. Together with the rise in pulmonary pressures, a modest increase of RV wall thickness was found, indicating mild RV hypertrophy at start of training.

At this time point, there were no signs of cardiac dysfunction or adverse remodeling, measured by cardiac output, stroke volume, heart rate, TAPSE, or RVEDD (Table S-1).

#### Induction of stable vs. progressive PH by different monocrotaline dosages

Serial echocardiographic measurements revealed different phenotype of PH induced by MCT 40 or 60 mg/kg (Figure S-1).

**Figure S2.1** Two distinct phenotypes of pulmonary hypertension (PH) were induced by the use of a low (40 mg/kg) and a high dose of monocrotaline (60 mg/kg). In stable PH (MCT40 - untrained rats), from day 14 after MCT-injection, there was no significant further increase in PVR and resting cardiac output was preserved ( $\Delta\text{PVR} = +7.5 \pm 5.0 \text{ \%/day}$ ,  $\Delta\text{cardiac output} = -0.74 \pm 0.81 \text{ \%/day}$ , cardiac output at end =  $109 \pm 10 \text{ ml/min}$ ). In progressive PH (MCT60 - untrained rats), a rapid increase in PVR and a marked decline in cardiac output were seen ( $\Delta\text{PVR} = +11 \pm 4 \text{ \%/day}$ ,  $\Delta\text{cardiac output} = -3.1 \pm 0.8 \text{ \%/day}$ , cardiac output at end =  $69 \pm 10 \text{ ml/min}$ ).  $\Delta\text{PVR}$  and  $\Delta\text{cardiac output}$  are hemodynamic parameters for disease progression and correspond with the slope indicated in the figure. They were calculated as stated in the methods section of the main article, the numeric data can be found in Table Suppl-2. All data are presented as mean  $\pm$  SEM.



In stable PH-Sed (MCT40 - untrained rats), from day 14 after MCT-injection, there was no significantly further increase in PVR, and resting cardiac output was preserved ( $\Delta\text{PVR} = +7.5 \pm 5.0 \text{ \%/day}$ ,  $\Delta\text{CO} = -0.74 \pm 0.81 \text{ \%/day}$ , cardiac output at end =  $109 \pm 10 \text{ ml/min}$ ). Nevertheless, some signs of RV dysfunction and adverse remodeling were observed in stable PH-Sed at end of study protocol (TAPSE, stable PH-Sed:  $2.9 \pm 0.3 \text{ mm}$  vs. control-Sed:  $3.7 \pm 0.1 \text{ mm}$ ; RVEDD, stable PH-Sed:  $5.4 \pm 0.4 \text{ mm}$  vs. control-Sed:  $3.5 \pm 0.1 \text{ mm}$ ; all  $p < 0.05$ ).

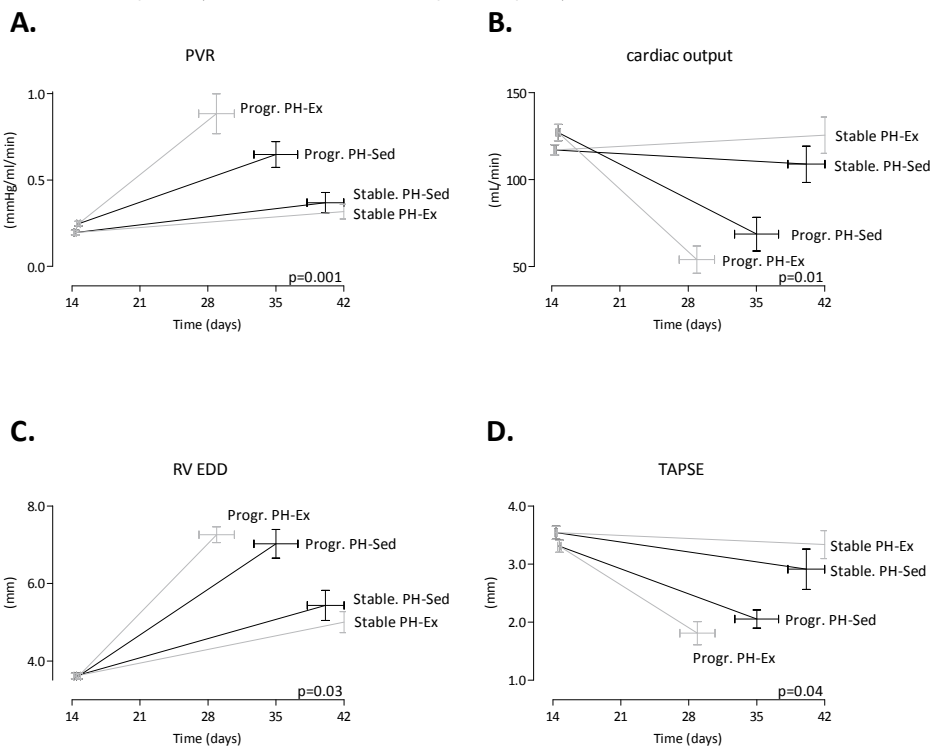
In progressive PH-Sed (MCT60 - untrained rats), a rapid increase in PVR and a marked decline in cardiac output were seen ( $\Delta\text{PVR} = +11 \pm 4 \text{ \%/day}$ ,  $\Delta\text{CO} = -3.1 \pm 0.8 \text{ \%/day}$ , cardiac output at end =  $69 \pm 10 \text{ ml/min}$ ; all  $p < 0.05$  vs. stable PH-Sed).

#### Effects of training on disease progression in stable vs. progressive PH

Serial echocardiographic measurements were used to study the effect of training on disease progression in control, stable and progressive PH.

**Figure 2.2** Effect of exercise training on disease progression

Opposite effects of training in stable vs. progressive PH were found for all important hemodynamic parameters for disease progression (indicated by the slope of the connecting lines from “start of training” to “end of study protocol”). All data are presented as mean $\pm$ SEM. p-values represent the interactive effect per individual parameter. Each line corresponds with an experimental group (see bottom). The control group was omitted for clarity. Numeric data at start of training are found in Table Suppl-1, numeric data on disease progression are found in Table Suppl-2. Abbreviations: PVR = pulmonary vascular resistance; TAPSE = tricuspid annular plane systolic excursion; RVEDD = RV end diastolic diameter.

**Table S2-2** Effect of training on disease progression

	Control		Stable PH		Progressive PH		Interaction
	Sed	Ex	Sed	Ex	Sed	Ex	p-value
Disease progression (during exercise period)							
ΔCO (%/day)	+0.40±0.20	+0.24±0.26	-0.74±0.81	+0.20±0.32	-3.1±0.8	-5.8±1.0 **	0.01
ΔSV (%/day)	+0.59±0.18	+0.43±0.24	-0.05±0.60	-0.68±0.23	-2.2±0.8	-4.9±1.0 **	0.02
ΔHR (%/day)	-0.17±0.06	-0.18±0.08	-0.81±0.39	-0.44±0.14	-0.88±0.22	-1.8±0.3 *	0.02
ΔTAPSE (%/day)	+0.05±0.10	+0.07±0.08	-1.1±0.8	-0.27±0.30	-2.5±0.6	-4.2±0.6 *	0.04
ΔRVWT (%/day)	+0.01±0.11	-0.11±0.12	+1.4±0.3	+0.5±0.2	+1.2±0.4	+2.5±0.4 **	<0.001
ΔRVEDD (%/day)	-0.13±0.11	+0.18±0.16	+2.7±1.0	1.6±0.3	+6.6±1.5	+10±1 *	0.03
ΔRVSP (%/day)	+0.68±0.50	0.37±0.52	+2.8±1.0	+2.7±0.6	+2.0±0.7	+6.6±2.1 *	0.04
ΔPVR (%/day)	+0.76±0.70	+0.26±0.58	+7.5±5.0	+2.7±0.9	+11±4	+35±9 **	0.001

Opposite effects of training on disease progression in stable vs. progressive PH were observed for all parameters. All data are presented as mean $\pm$ SEM. \*:  $p < 0.05$ , \*\*:  $p < 0.01$  progressive PH-Ex vs. pPH-Sed. Abbreviations:  $\Delta$ CO ( $\Delta$ SV,  $\Delta$ HR, ...) = daily percentage change of cardiac output, stroke volume, and so on, during exercise period.

Figure 2 shows the evolution of the various echo-derived hemodynamic parameters over time for the different experimental groups (numeric data: Table S-2). The divergent effect of training on disease progression is especially evident for cardiac output (Figure 2B). During the exercise period, a steeper fall in cardiac output was observed for progressive PH-Ex vs. progressive PH-Sed, whereas cardiac output was better preserved in stable PH-Ex than stable PH-Sed.

#### Effects of training on survival and endurance in stable vs. progressive PH

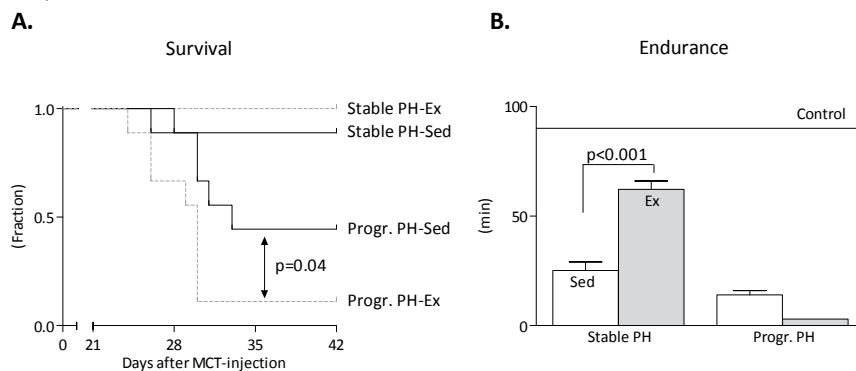
Loss in body mass and decline in cardiac output were closely correlated ( $r = 0.74$ ,  $p < 0.001$ ), which verifies the clinical criteria that were used for right heart failure. Based on these criteria, we observed that exercise training decreased survival significantly in progressive PH (hazard ratio, progressive PH-Ex vs. progressive PH-Sed = 2.7, 95%CI: 1.1-14.2); In stable PH-Sed, one rat prematurely developed right heart failure, all rats in stable PH-Ex and the control groups survived (Figure 3A).

At four weeks, training significantly improved exercise endurance by twofold in stable PH (endurance, stable PH-Ex:  $62 \pm 4$  min vs. stable PH-Sed:  $25 \pm 4$  min,  $p < 0.001$ ), whereas an opposite trend was observed in progressive PH. A strong significant interaction between training-status and PH-status was present (Figure 3B;  $p < 0.001$ ).

The opposite effect of training on survival and endurance are in line with the previous findings on disease progression.

#### Figure 2.3 Effects of exercise training on survival and endurance

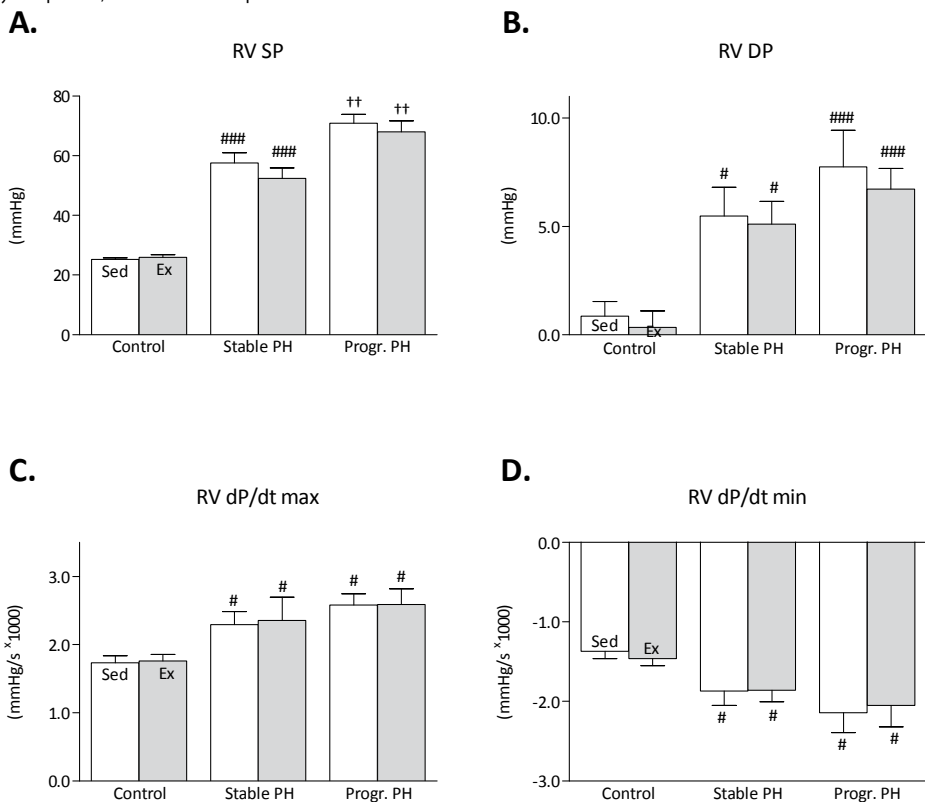
Training had a detrimental effect on survival in progressive PH, whereas it did not affect survival in stable PH (A). All rats from control groups survived, and were omitted here for clarity. Training improved endurance more than twofold in stable PH, whereas a trend for an opposite effect was observed in progressive PH (B; interactive effect:  $p < 0.001$ ). All rats in the control groups reach the predefined maximum endurance time (90 min, indicated by the horizontal line). All data are presented as mean  $\pm$  SEM. Endurance testing was only performed on surviving rats, therefore:  $n=10$  (control-Sed),  $n=10$  (control-Ex),  $n=8$  (stable PH-Sed),  $n=9$  (stable PH-Ex),  $n=4$  (progressive PH-Sed),  $n=1$  (progressive PH-Ex).



## RV catheterization at end of study protocol

RV catheterization at end of study protocol confirmed the pulmonary hypertensive status of MCT-treated rats (Figure S-2). There was a strong MCT-dose dependent response at end of study protocol ( $p < 0.001$ ): RVSP at rest doubled in stable PH and almost tripled in progressive PH vs. control, and a similar pattern was seen for RV diastolic pressures. In addition, the intrinsic RV contractility and relaxation were significantly changed in stable and progressive PH vs. control. However, no (statistical) differences in RV dP/dt max and RV dP/dt min were found between the two PH-phenotypes. RV catheterization at end of study protocol revealed no (interactive) effect of training.

**Figure S2.2** RV catheterization at the end of study confirmed the pulmonary hypertensive status of the sedentary and trained stable and progressive PH rats. Although the intrinsic RV contractility and RV relaxation were significantly altered in stable and progressive PH vs. control, rest-measurements did not reveal differences among the two PH-phenotypes. No (interactive) effect of training was observed. All data are presented as mean  $\pm$  SEM. #:  $p < 0.05$ , ##:  $p < 0.01$ , ###:  $p < 0.001$  vs. control; ††:  $p < 0.01$  vs. stable PH (and  $p < 0.001$  vs. control). RV SP = RV systolic pressure; RV DP = RV diastolic pressure.



## Effects of training on pulmonary vascular remodeling in stable vs. progressive PH

Training increased (wet) lung weights in progressive PH ( $p<0.001$ ), whereas it had no effect on lung weights in stable PH, independent of normalization (i.e. normalization by body mass or tibia length; Figure 4A, Table S-3). Moreover, a strong interaction between training-status and PH-status was present ( $p<0.001$ ). Wet/dry lung mass ratios were similar for all experimental groups (wet/dry ratios between groups varied from  $4.9\pm 0.1$  to  $5.2\pm 0.1$ , see Table S-3). This suggests that training modulated pulmonary vascular remodeling, and that the observed differences are not likely to be attributed to pulmonary edema. Measurements on PA wall thickness confirmed the interactive effect of training on pulmonary vascular remodeling (Figure 4B). Echo-derived PVR at end of study protocol and PA wall thickness correlated well ( $r = 0.82$ ,  $p<0.001$ ), indicating that the pulmonary vascular remodeling must have had profound hemodynamic effects *in vivo*.

**Table S2-3** Autopsy data

	Control		Stable PH		Progressive PH		Interaction
	Sed	Ex	Sed	Ex	Sed	Ex	p-value
Autopsy							
Body mass (g)	404±11	390±14	394±14	398±5	327±16	308±7	0.63
BM change (%/2d)	+1.6±0.4	+1.0±0.4	+0.8±0.9	+1.1±0.2	-4.2±2.1	-7.3±2.0	0.21
Lung mass (wet) (g)	1.47±0.05	1.42±0.07	1.95±0.08	1.68±0.09	2.23±0.14	2.88±0.15 ***	<0.001
Lung wet / dry mass ratio	5.0±0.1	4.9±0.1	5.2± 0.2	5.1±0.1	5.2±0.1	5.0±0.2	0.98
Heart mass (g)	1.42± 0.06	1.48±0.07	1.67±0.09	1.67±0.09	1.69±0.05	1.70±0.09	0.91
RV mass (g)	0.28±0.02	0.31±0.01	0.55±0.04	0.46±0.03	0.57±0.04	0.56±0.02	0.09
LV mass (+septum) (g)	0.98±0.05	1.01±0.05	0.93±0.04	0.96±0.03	0.79±0.03	0.76±0.04	0.69
RV / (LV + S) (g/g)	0.29±0.02	0.31±0.02	0.60±0.05	0.48±0.04	0.72±0.04	0.76±0.05	0.11
Liver (g)	14.5±0.6	13.9±0.8	15.0±0.6	15.2±0.6	11.3±1.0	10.2±0.4	0.65
Kidneys (g)	2.4±0.2	2.5±0.1	2.6±0.1	2.5±0.1	2.0±0.1	2.1±0.1	0.47
Spleen (g)	0.74±0.04	0.72±0.04	0.84±0.06	0.79±0.04	0.69±0.05	0.69±0.03	0.89
Brains (g)	2.02±0.03	1.96±0.05	1.99±0.02	1.96±0.04	1.88±0.05	1.93±0.04	0.31
Tibia length (mm)	37.0±0.3	37.0±0.3	37.0±0.3	38.0±0.4	36.0±0.3	36.0±0.3	0.34

A strong interactive effect Training \* PH was observed for (wet) lungs mass only. This interactive effect remained strongly significant independent of normalization (i.e. normalization by body mass or tibia length), and was not attributed to edema (the lung wet/dry ratio did not differ between the experimental groups). A strong MCT-dose dependent response was observed ( $p<0.001$  for all parameters, except brain mass). All data are presented as mean±SEM (wet weights). \*\*\*:  $p<0.001$  progressive PH-Ex vs. progressive PH-Sed. Abbreviations: BM change = relative body mass change over the last two days; RV / (LV + S) = RV over LV (including septum) mass ratio.

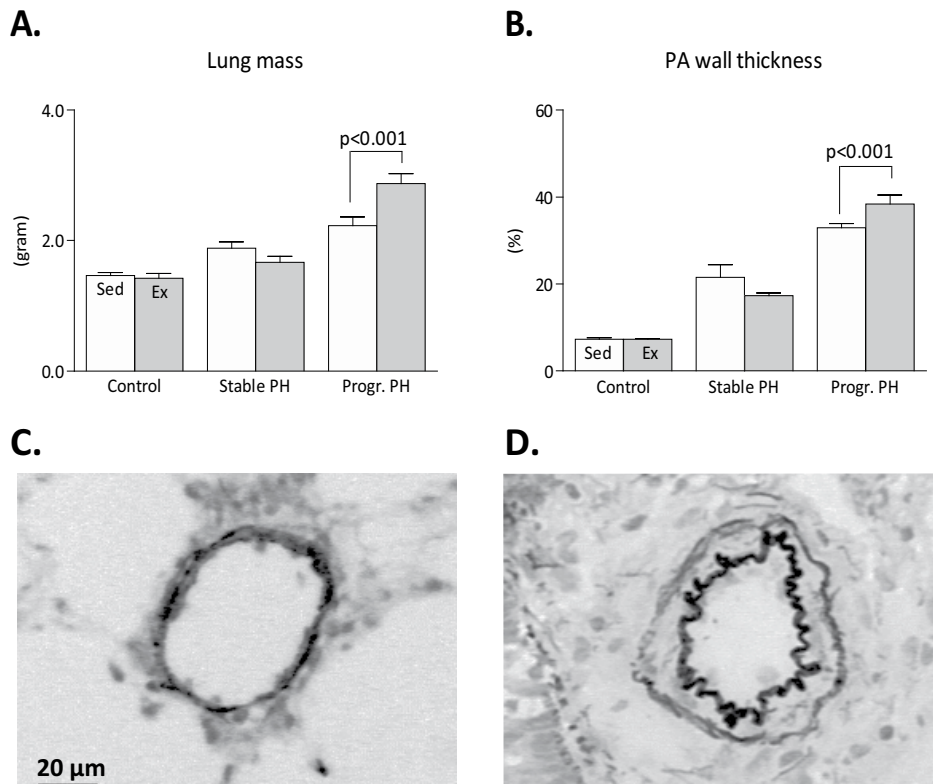
## Effects of training on cardiac remodeling in stable vs. progressive PH

*Histomorphology:*

In contrast with pulmonary vascular remodeling, the amount of RV hypertrophy was similar among stable and progressive PH, whether it was expressed by RV mass (normalized or not) or RV/(LV+septum) (Table S-3), confirming echocardiographic observations. Measurements of RV cardiomyocyte CSA also showed similar RV hypertrophy between stable and

**Figure 2.4** Effects of exercise training on pulmonary vascular remodeling

Training significantly worsened pulmonary vascular remodeling in progressive PH, demonstrated by elevated (wet) lung mass and increased PA wall thickness in progressive PH-Ex, compared to progressive PH-Sed ( $p<0.001$ ). Moreover, significant interactions between training-status and PH-status were present (A,B;  $p<0.001$  for lung mass,  $p<0.01$  for PA wall thickness). Lower panels show typical examples of pulmonary arteries (Elastica von Giesson, 200x magnification) of control-Sed (C) and progressive PH-Ex (D). Data are presented as mean $\pm$ SEM.



progressive PH, compared to control (Figure 5A). RV fibrosis was significantly increased in progressive PH only; no additional effect of training was found (Figure 5B). Similar levels of RV hypertrophy but differences in RV diameters (Figure 5C) indicate concentric vs. eccentric remodeling, which translated to severely elevated RV wall stress levels for progressive PH, but only moderately elevated wall stress levels for stable PH; no interactive effect of training was observed (Figure 5D).

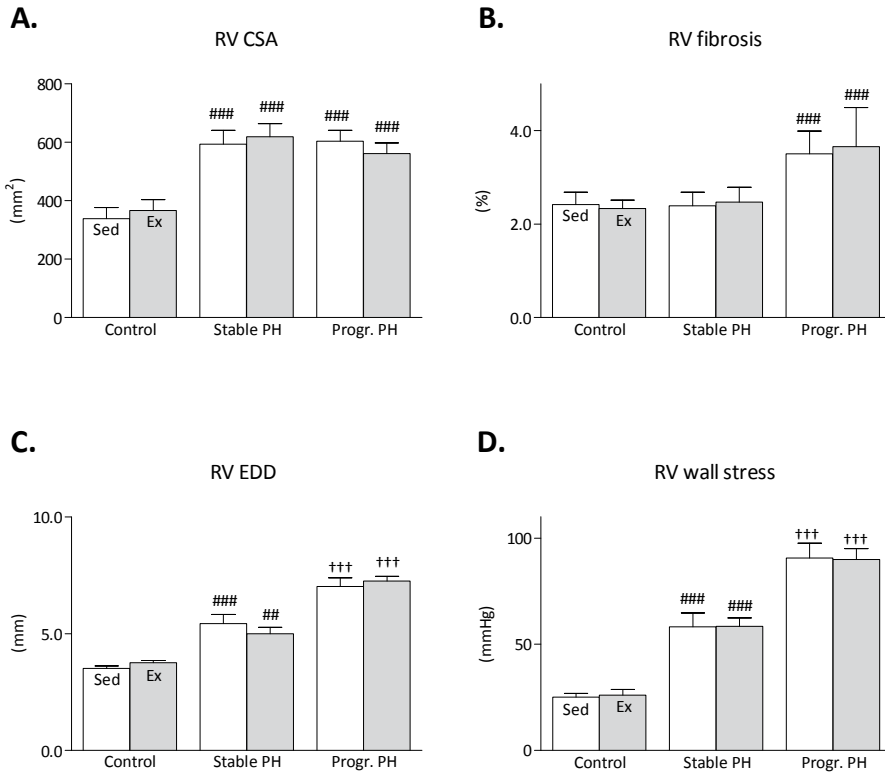
Analyses of LV myocardium revealed atrophy (evident from lower LV mass and lower LV cardiomyocyte CSA) and increased fibrosis in progressive PH only. No effect of training was observed on LV myocardium in progressive PH (Figure S-3).

#### *Cardiac capillarization and inflammation:*

Training improved RV capillarization in stable PH with approximately +25%, whereas a trend for an opposite effect was seen in progressive PH, whether expressed as a ratio of capillaries

**Figure 2.5** Effects of exercise training on right ventricular remodeling

The amount of RV hypertrophy indicated by RV CSA was similar in stable and progressive PH (A). RV fibrosis was only seen in progressive PH, and no (interactive) effect of training was observed (B). The largest RV diameters were observed in progressive PH (C) translating into the highest RV wall stress (D). All data are presented as mean $\pm$ SEM. ##:  $p<0.01$ , ###:  $p<0.001$  vs. control. †††:  $p<0.001$  vs. stable PH (and  $p<0.001$  vs. control).

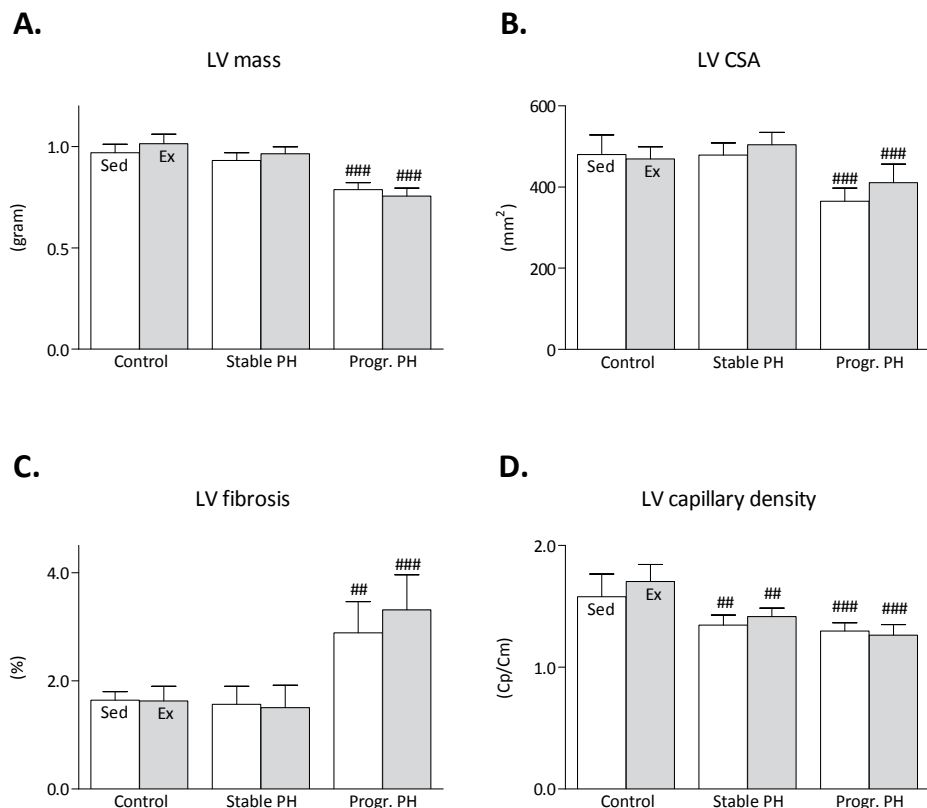


per cardiomyocyte or as capillaries per section area (Figure 6A,B). Moreover, a significant interaction between training-status and PH-status was present ( $p<0.01$ ). Training had no effect on capillarization in LV (Figure S-3D).

In progressive PH, clusters of leukocytes were observed in various parts of the myocardium of the right ventricle (Figure 7D). As a result, the number of leukocytes was significantly higher in progressive PH, compared to control. Training dramatically increased RV leukocyte infiltration in progressive PH, whereas in stable PH it remained unchanged (Figure 7A). Moreover, a very significant interaction between training-status and PH-status was present ( $p<0.001$ ). Leukocyte infiltration was only observed in the right ventricle. Analysis of LV showed no differences between groups, their values were even slightly lower than RV control values (Figure 7B).



**Figure S2.3** LV atrophy (evident from lower LV mass and lower LV CSA) and LV fibrosis was observed in progressive pulmonary hypertension only. LV capillarization was decreased in both stable and progressive pulmonary hypertension in comparison with control. Training had no effect on LV morphology in both stable and progressive pulmonary hypertension. All data are presented as mean $\pm$ SEM. ##:  $p<0.01$ ; ###:  $p<0.001$  vs. control. Cp/Cm = number of capillaries per cardiomyocyte.



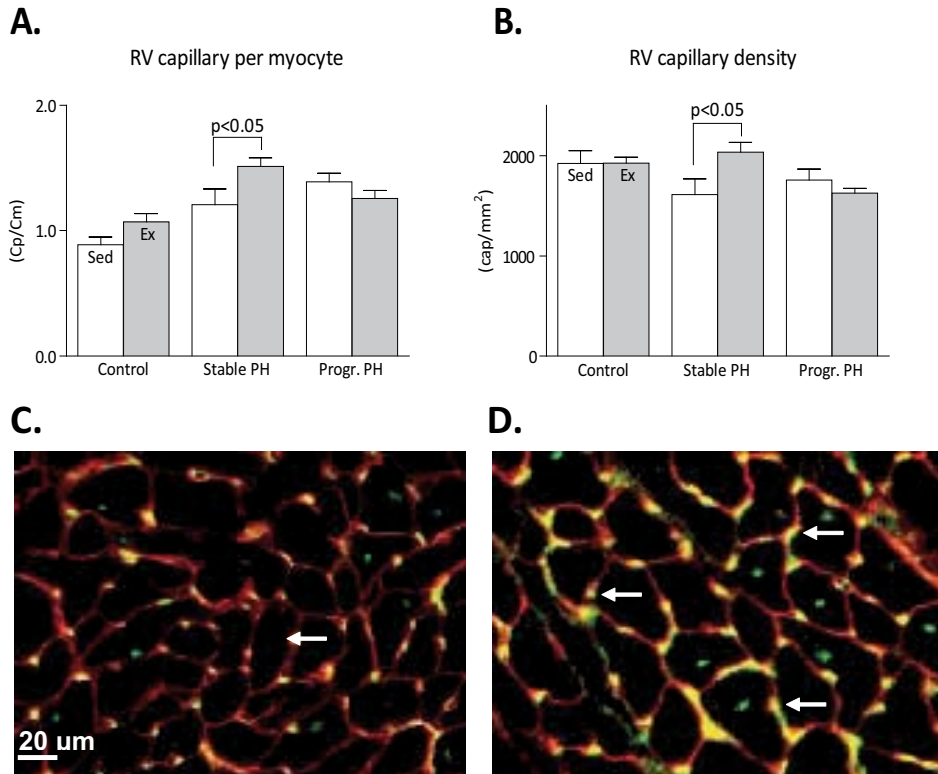
## DISCUSSION

To the best of our knowledge, this is the first study that investigated the effects of training in stable and progressive PH, focusing on RV function and remodeling. Using a comprehensive set of physiologic and pathologic endpoints, we have demonstrated that:

- 1) Exercise training was well tolerated and beneficial in PH with a preserved cardiac output. In this group, training had no adverse effects on disease progression; it improved endurance, and was associated with enhanced RV capillarization. However,
- 2) Exercise training was detrimental in progressive PH developing right heart failure. Here, exercise had adverse effects on hemodynamics and accelerated the progression to right heart failure. Moreover, exercise was associated with adverse pulmonary vascular remodeling, and massive RV inflammation.

**Figure 2.6** Effects of exercise training on right ventricular capillarization

Training improved RV capillarization in stable PH, whereas a trend for an opposite effect was observed in progressive PH (capillarization expressed as capillary-to-cardiomyocyte ratio or Cp/Cm (A) or capillary per area (B)). Moreover, a significant interaction between training-status and PH-status was present ( $p < 0.01$ ). Lower panels show typical examples of RV capillarization (immunofluorescence CD31, 100x magnification) of control-Sed (C) and stable PH (D). Green = CD31, red = cardiomyocyte cell membranes; capillaries can be identified as green / yellow dots (merging of red and green; arrow). All data are presented as mean  $\pm$  SEM.



### Functional improvement after training, associated with enhanced capillarization

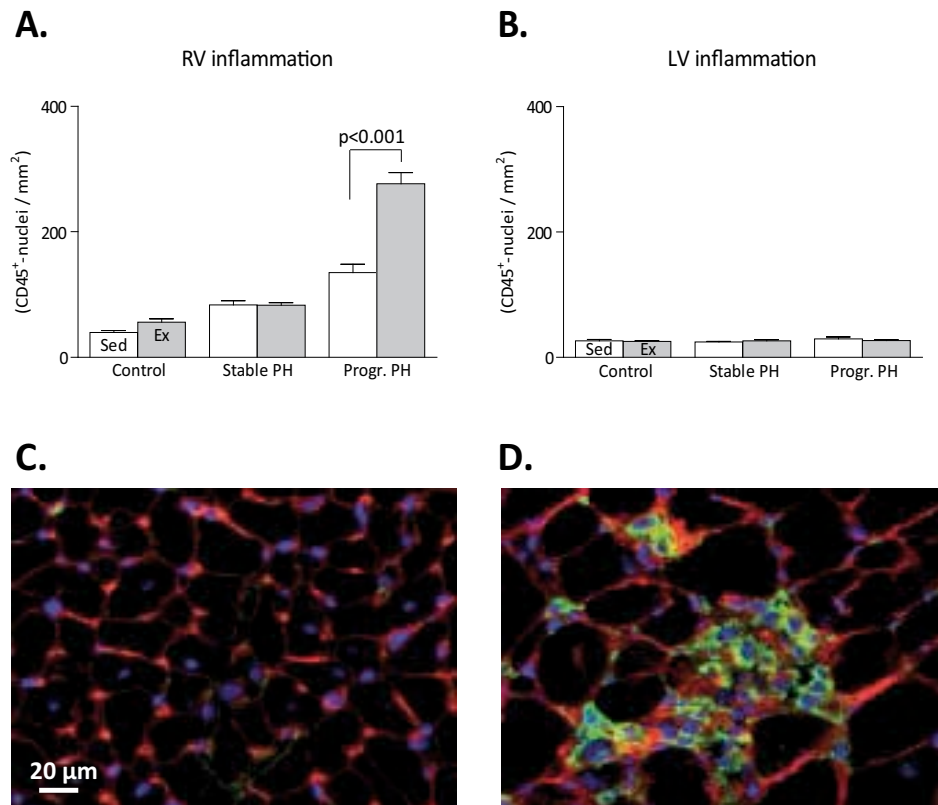
The only prospective clinical trial on exercise training in PH, by Mereles *et al*, showed that functional capacity and quality of life of stable PH-patients could markedly be improved after training.<sup>7</sup> The general hemodynamic characteristics of the subjects in that trial were similar to the stable PH-group in our study: comparable pulmonary artery pressures were found, together with a mildly depressed cardiac index at rest, and moderate RV dilatation that remained stable during the study period. In agreement with this clinical study we found a marked improvement in exercise endurance in stable PH after training.

In addition, we found that in stable PH the functional improvement after training was associated with enhanced capillarization of the right ventricle. This phenomenon has been described for ischemic heart failure,<sup>20</sup> and recently two studies have evaluated the effect of training on cardiac angiogenesis in systemic hypertension as well.<sup>21,22</sup> In spontaneous hypertensive rats<sup>21</sup> and in rats with angiotensin II-induced hypertension,<sup>22</sup> exercise was found to

**Figure 2.7** Effects of exercise training on leukocyte infiltration

Training had a dramatic effect on RV leukocyte infiltration in progressive PH, whereas leukocyte infiltration remained unchanged in stable PH (A). Moreover, there was a strong significant interaction between training-status and PH-status ( $p < 0.001$ ).

Inflammation was restricted to the right ventricle only, as values for leukocyte infiltration of LV did not differ among the experimental groups and were even lower than RV control values (B). Lower panels show typical examples of RV leukocyte infiltration (immunofluorescence CD45; 100x magnification) of control-Sed (C) and progressive PH-Ex (D). Green = CD45, blue = nuclei; red = cardiomyocyte cell membranes; notice the clustering of aggregated leukocytes in progressive PH-Ex into the myocardium of the right ventricle. Data are presented as mean  $\pm$  SEM.



improve (LV) capillarization by about 40%. This is somewhat higher than the 25% increase in (RV) capillarization observed in our present study, but the difference may be explained by the lower training intensity and shorter exercise period in our study. A direct link between angiogenesis, hypertrophy and cardiac function has been shown.<sup>23,24</sup> Insufficient cardiac microvascular growth was recently identified as an important underlying mechanism in the transition from compensatory hypertrophy to heart failure.<sup>23</sup> Moreover, promotion of cardiac angiogenesis was found to normalize the relative capillary deficit, to improve coronary flow reserve, and to restore cardiac dysfunction under chronic pressure overload.<sup>24</sup>

It is likely that the improved endurance in stable PH is also partially attributable to other beneficial effects of exercise that were not studied here. Especially, its effects on skeletal

muscle function in PH deserves further exploration in future studies, as this effect was shown to be relevant in the rehabilitation of (left) heart failure- and COPD-patients.<sup>25;26</sup>

#### Worsened survival after training, associated with enhanced RV inflammation

Traditionally, PH-patients were encouraged to limit physical activity, a view that was mainly based on theoretical arguments<sup>3</sup>. Here, we demonstrate that exercise training in progressive PH can indeed be harmful in the case of a poorly adapted right ventricle, by augmentation of pressure overload associated RV inflammation.

It is unlikely that RV inflammation is primarily the result of a direct inflammatory effect of MCT on the heart.<sup>27</sup> We found no evidence for LV inflammation, even in rats that were treated with the highest MCT-dose. Furthermore, histology of the right ventricle revealed randomly distributed patches of infiltration, rather than a gradual pattern of inflammatory cells diffusing from the (sub)endocardium. For these reasons, RV inflammation in our model is most likely ascribed to chronic RV pressure overload.

To the best of our knowledge, the link between RV inflammation and chronic RV pressure overload has not been investigated yet, neither clinically nor experimentally. Nevertheless, in patients suffering from acute pulmonary embolism (APE), comparable observations of selective RV inflammation were reported in a postmortem study,<sup>18</sup> and similar findings were observed in experimentally induced APE.<sup>28</sup> The mechanistic importance of RV inflammation was demonstrated, since suppression of the inflammatory response following APE limited RV damage and prevented right heart failure.<sup>29</sup> In these studies, it was suggested that RV inflammation could have been triggered by ischemic injury of the right ventricle or local and/or systemic over-production of catecholamines.

High RV wall stress might be an alternative explanation, as recently shown in a model of chronic LV pressure overload.<sup>9;30</sup> We observed a similar amount of RV hypertrophy in all PH-groups. However, because of larger RVEDD, we found the highest RV wall stress in progressive PH. At resting conditions, RV wall stress was similar in progressive PH-Ex and progressive PH-Sed. Although we could not directly measure RV pressures during exercise, RV afterload probably increased significantly during exercise, because of the elevated PVR.<sup>8</sup> Therefore, it is likely that in progressive PH, RV wall stress was higher during exercise. These episodes of elevated wall stress could have triggered RV inflammation, because short periods of mechanical stretch (10 minutes) can induce myocardial over-expression of pro-inflammatory cytokines (like TNF- $\alpha$ ), which is followed by leukocyte infiltration.<sup>9;30</sup>

Finally, training might also directly aggravate pre-existing inflammation in the myocardium. In viral myocarditis it is known that exercise augments the inflammatory reaction, enhances cardiac dilatation, and increases its lethality.<sup>31;32</sup>

Our study suggests that RV inflammation in PH may be of pathophysiological importance. Future studies should investigate its relevance for the different etiologies of clinical pulmonary arterial hypertension.

### Limitations

The model of PH that was caused by the use of monocrotaline, does not fully replicate the pathophysiology and resulting pulmonary and cardiovascular effects of clinical PH. Therefore, this study should be viewed as a seminal analysis of exercise in stable and progressive PH from which other (clinical) studies should arise. For example, validated clinical determinants that can predict a favorable response to exercise training are currently absent.

The non-invasive estimation of PVR results from several measurements and is therefore susceptible to a large variability. Nevertheless, a close correlation was observed between echo-derived PVR measurements and histological parameters for pulmonary vascular remodeling.

Echo- or invasive hemodynamic measurements at end of study protocol failed to detect changes that could explain the differences in survival and endurance between the trained and sedentary groups. Echo- and invasive hemodynamic measurements were however obtained at rest and therefore do not reflect exercise hemodynamics, which probably differed between both groups. Moreover, in progressive PH, hemodynamic measurements at end of study protocol were obtained at a stage of terminal right heart failure, which was however reached earlier in the trained than the sedentary group. Hence, the differences in survival between both groups are reflected more by the time elapsed to reach right heart failure, than the hemodynamic findings at right heart failure.

### Conclusions

In our rat model, exercise training was found to be beneficial in stable PH, but detrimental in progressive PH. The differential effect is probably due to enhanced RV myocardial capillarization in stable PH, and RV myocardial inflammation in progressive PH. Future studies are necessary to address the clinical implications of our findings.

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# 3

## Effects of Exercise Training in Patients with idiopathic Pulmonary Arterial Hypertension



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*Eur Resp J* 2009; 34: 669-675

## ABSTRACT

*Objective* – We determined the physiological effects of exercise training on exercise capacity and quadriceps muscle function in patients with idiopathic pulmonary arterial hypertension (iPAH).

*Methods* – In total, 19 clinically stable iPAH patients (New York Heart Association II-III) underwent a supervised exercise training program for the duration of 12 weeks. Maximal capacity, endurance capacity, and quadriceps function were assessed at baseline and after twelve weeks. In 12 patients, serial quadriceps muscle biopsies were obtained.

*Results* – Six minute walk distance and peak exercise capacity did not change after training. However, endurance capacity improved significantly after training, demonstrated by a shift of the anaerobic threshold to a higher workload (from  $32 \pm 5$  to  $46 \pm 6$  Watt;  $p=0.003$ ) together with an increase in exercise endurance time ( $p<0.001$ ). Moreover, exercise training increased quadriceps strength by 13% ( $p=0.005$ ) and quadriceps endurance by 34% ( $p=0.001$ ).

Training enhanced aerobic capacity of the quadriceps, by increasing capillarization ( $1.36 \pm 0.10$  to  $1.78 \pm 0.13$  capillaries per muscle fiber;  $p<0.001$ ) and oxidative enzyme activity, especially of the type-I (slow) muscle fibers. No changes were found in cross sectional area and fiber type distribution.

*Conclusions* – Exercise training in iPAH improves exercise endurance and quadriceps muscle function, which is also reflected by structural changes of the quadriceps.

## INTRODUCTION

Idiopathic pulmonary arterial hypertension (iPAH) is a life threatening disease, which eventually leads to right heart failure. A high pulmonary vascular resistance and right ventricular dysfunction impair stroke volume, thereby limiting oxygen supply to the skeletal muscles, especially during exercise, resulting in lactic acidosis at low work rates and impaired functional capacity.<sup>1,2</sup>

Traditionally, exercise training in iPAH patients was contraindicated due to the risk of sudden cardiac death.<sup>3</sup> However, with the increase in medical treatment options in the last decennium, the prognosis has improved significantly, and the role of exercise training in patients with iPAH was reconsidered.<sup>3</sup> Recently, the first clinical trial on exercise training in patients with pulmonary arterial hypertension reported promising results of improved exercise capacity and quality of life.<sup>4</sup>

Exercise training is a well-established adjunct therapy in several chronic diseases such as Chronic obstructive Pulmonary Disease (COPD) and congestive heart failure.<sup>5,6</sup> In patients with these chronic diseases, skeletal muscle dysfunction contributes to exercise intolerance.<sup>7-9</sup> The beneficial effects of exercise training in COPD and congestive heart failure are partially attributed to improved skeletal muscle efficiency<sup>10,11</sup> and increased capillary density and oxidative enzyme activity in quadriceps muscle biopsies.<sup>11-13</sup>

Also in iPAH patients, skeletal muscle dysfunction has been reported. Respiratory muscle dysfunction was found in two studies, both by voluntary and non-voluntary techniques.<sup>14,15</sup> More recently, forearm muscle dysfunction has been reported in these patients.<sup>16</sup> In a pilot study, examining voluntary strength of the respiratory, forearm and quadriceps muscles in iPAH patients, we previously found *specific* quadriceps muscle dysfunction.<sup>17</sup> However, these studies focused on muscle function only, without giving more insight in the roles of muscle atrophy, fiber type switching, decreased oxidative enzyme activity, or reduced capillary density, as potential underlying mechanisms of skeletal muscle dysfunction.<sup>18</sup>

We hypothesized that exercise training in iPAH patients improves exercise capacity and diminishes quadriceps muscle dysfunction by counteracting these structural muscle alterations. We therefore assessed the effects of an outpatient exercise training program on exercise capacity, quadriceps function and quadriceps structure.

## METHODS

### Study population

In total, 19 patients were recruited from the VU University Medical Center (Amsterdam, The Netherlands) between 2006 and 2008 and met the following criteria: 1) Diagnosed with iPAH according to World Health Organization criteria<sup>19</sup> established by right heart catheterization;

2) Stable clinical condition, defined as a change in six minute walk distance (6MWD) of less than 10% in three consecutive measurements prior to inclusion (over a period of minimally one year), and no change in medical therapy for at least three months; 3) Aged 18 years or older; 4) Living within five kilometers of a rehabilitation center associated with this study.

The Institutional Review Board on Research Involving Human Subjects (Amsterdam, The Netherlands) approved the protocol. Informed consent was obtained from all subjects.

### Study protocol

Patients were evaluated at baseline and after 12 weeks, on two consecutive days. On day 1: a cardio-pulmonary exercise test (CPET), quadriceps function tests and pulmonary function tests<sup>20</sup> were performed, and N-terminal pro-B-type natriuretic peptide (NT-proBNP) was determined. On day 2, an endurance exercise test was performed and the 6MWD was determined. In addition, 12 out of 19 patients underwent a quadriceps muscle biopsy on the second day of evaluation before and after training.

All patients attended an exercise training program of three times a week in a rehabilitation center for a period of 12 weeks. The training program was performed in rehabilitation centers nearby, according to usual clinical care. The standardized exercise protocol was adopted from the American Heart Association guidelines for rehabilitation of chronic heart failure patients.<sup>5</sup> The exercise training consisted of cycling and quadriceps muscle training (Table 1). For safety reasons, physiotherapists, who recorded heart rate and oxygen saturation, always accompanied the patients. When oxygen saturation dropped below 85%, or heart rate exceeded 120 bpm, the training session was paused or terminated earlier. Rehabilitation

**Table 3.1** 12-week supervised exercise training protocol

Week	Intensity	Exercise	Rest time	Sets
<b>Cycle exercise</b>				
1-3	50% $\text{VO}_{2\text{max}}$	2 min	2 min	10
4-6	50% $\text{VO}_{2\text{max}}$	3 min	2 min	7
7-9	75% $\text{VO}_{2\text{max}}$	4 min	2 min	6
10-12	75% $\text{VO}_{2\text{max}}$	5 min	2 min	5
<b>Quadriceps strength</b>				
1-3	50% ORM	12 rep	1 min	3
4-6	50% ORM	13 rep	1 min	3
7-9	75% ORM	14 rep	1 min	3
10-12	75% ORM	15 rep	1 min	3
<b>Quadriceps endurance</b>				
1-3	30% ORM	30 rep	1 min	3
4-6	30% ORM	40 rep	1 min	4
7-9	40% ORM	50 rep	1 min	5
10-12	40% ORM	60 rep	1 min	6

The exercise training consisted of cycle training (based on  $\text{VO}_{2\text{max}}$  assessed at baseline measurements) and quadriceps training (based on one-repetition-maximum assessed at first day of training). Abbreviations:  $\text{VO}_{2\text{max}}$ : maximal oxygen consumption determined during CPET; ORM: one-repetition-maximum; rep: repetition

physicians or pulmonologists were on site and directly available for consultation. Attendance was recorded as an indicator for patient's compliance.

#### Exercise testing

6MWD test was performed according to American Thoracic Society guidelines.<sup>21</sup> Maximal capacity was determined during CPET as described previously.<sup>22</sup> During CPET heart frequency, pulse oximetry and gas exchange (breath-by-breath) were recorded. Anaerobic threshold was determined by the V-slope method.<sup>23</sup>

Endurance capacity was evaluated by a submaximal exercise test performed at a constant load of 75% of baseline peak-workload.<sup>24</sup> After 3 minutes of rest and 3 minutes of unloaded cycling, the patients had to exercise at 75% of baseline peak workload for as long as possible or the observer terminated the test after 15 minutes. During the submaximal test heart frequency, pulse oximetry and gas exchange (breath-by-breath) were recorded.

#### Quadriceps muscle function and biopsy

Quadriceps function was assessed with a hydraulic dynamometer, as previously described.<sup>25</sup> In addition, 12 out of 19 patients gave informed consent for a quadriceps muscle micro-biopsy at baseline and after training, to analyze the effects of training on structural changes of the quadriceps muscle. Circumference of the leg was measured. Under local anesthetics (2% lidocaine), biopsies were taken from the vastus lateralis of the quadriceps muscle 10 cm above the patella with a 16G spring-loaded biopsy needle (QC-16-15.0-10T, Cook Medical, Limerick, Ireland). The biopsy was immediately evaluated under the microscope, embedded in 15% gelatin in Tyrode's solution containing 20 mM butanedione monoxime and frozen in liquid nitrogen. Serial cryosections were cut at -20 °C and collected on slides coated with Vectabond (Vector Laboratories, Burlingame, CA, USA).

#### Number of capillaries in quadriceps muscle

Capillarization of the quadriceps muscle was determined by quantitative immunofluorescence microscopy. Briefly, quadriceps cryosections (5 µm) were incubated for 60 min with primary CD31-antibody (1:100; sc-1506-R, Santa Cruz Biotechnology, Santa Cruz, CA, USA), followed by secondary antibody staining with anti-rabbit Alexa fluor 488 (Vector Laboratories), as well as wheat germ agglutinin (WGA, glycocalyx; Molecular Probes, Invitrogen, Paisley, UK) and 4',6'-diamidino-2-phenylindole (DAPI, nuclei; Molecular Probes, Invitrogen) counterstaining. Image acquisition was performed on a Marianas digital imaging microscopy workstation (Intelligent Imaging Innovations (3i), Denver CO, USA) using multiple fluorescence channels. SlideBook imaging analysis software (SlideBook 4.2, 3i) was used to semi-automatically quantify the images. Capillarization was expressed as the number of capillaries per quadriceps myocyte measured in the whole section.

#### Fiber type distribution, oxidative enzyme activity, and cross sectional area

Cross sectional area (CSA), fiber type distribution and oxidative enzyme activity were determined as previously described.<sup>26,27</sup> In detail, in 10 randomly selected type I and 20 randomly selected type II cells (identified by serial sections stained for myofibrillar ATPase), oxidative enzyme activity was analyzed by measuring succinate dehydrogenase (SDH) absorbance at 660 nm (10  $\mu$ m sections incubated for 20 min at 37 °C). For the same cells, CSA were measured. Fiber type distribution was analyzed by counting of all type I and type II muscle fibers in the biopsy. Images were analyzed using ImageJ imaging analysis software (ImageJ for Windows 1.39a, NIH, Bethesda, MD, USA).

#### Statistics

All data are presented as mean $\pm$ SEM, unless stated otherwise. A p-value <0.05 was considered statistically significant. The effects of training on exercise capacity, quadriceps function and quadriceps capillarization were assed by a paired t-test. Changes in fiber type distribution were assessed by two-way repeated measures ANOVA. Oxidative enzyme activity and muscle fiber CSA were analyzed by multi-level analyses, to correct for the non-independence of successive measurements per patient.<sup>28</sup> Regression analyses were performed to study the association between changes in quadriceps endurance and aerobic capacity. Analyses were performed with SPSS 16.0 (SPSS Inc., Chicago, IL, USA) or MLwiN 2.02 software (University of Bristol, Bristol, UK).

## RESULTS

In total, 19 patients were recruited for the present study and their baseline characteristics are presented in Table 2. The patient cohort was typical iPAH, with a female predominance, median New York Heart Association class III, and a mean age of 42 years. In addition, the last known catheterization data (< 1 year) prior to the rehabilitation are given in Table 2.

The training program was well tolerated by all patients and no adverse events were observed. The compliance to the exercise training program was 91 $\pm$ 2%. Only two patients required a minor training adjustment, because of complaints of dizziness during the quadriceps exercise. Training did not elevate NT-proBNP levels (947 $\pm$ 429 to 1043 $\pm$ 462 pg/ml; n.s.) and did not change pulmonary function (data not shown).

#### Exercise training improved endurance

The CPET revealed no significant improvements of maximal capacity and gas-exchange after training. Moreover, exercise training did not improve 6MWD (Figure 1A). However, endurance exercise characteristics improved significantly after training, demonstrated by a shift

**Table 3.2** Patient characteristics at baseline

	Study population (n=19)
Gender male/female	4/15
Age (yr)	42±13
Height (m)	1.70±0.09
Weight (kg)	72±14
NYHA class II/III	3/16
NT-proBNP (pg/ml)	947±1606
<b>Hemodynamics</b>	
mPAP (mmHg)	53±19
PVR (dynes.s/cm <sup>2</sup> )	845±460
Cardiac index (l/min/m <sup>2</sup> )	2.7±0.9
Right atrial pressure (mmHg)	7±4
PCWP (mmHg)	5.7±2.4
<b>Exercise capacity</b>	
6 minute walk distance (m)	496±108
Peak work (Watt)	78±42
Peak VO <sub>2</sub> (ml/kg/min)	15±4
Peak VE (l/min)	63±30
Peak VECO <sub>2</sub>	51±13
<b>Medication</b>	
Single / combination treatment	8/11
Treatment duration (months)	20±16

Data are presented as mean±SD. BSA: body surface area; NYHA class: New York Heart Association functional class; pred: predicted value; mPAP: mean pulmonary artery pressure; PVR: pulmonary vascular resistance; PCWP: pulmonary capillary wedge pressure

of anaerobic threshold to a higher workload (from 32±5 to 46±6 Watt;  $p=0.003$ ). In addition, exercise endurance time increased with 89% after training (Figure 1B;  $p<0.001$ ).

#### Exercise training improved quadriceps function and aerobic capacity

Quadriceps muscle strength improved modestly with 13% after training (Figure 1C; from 94±7 to 106±8 Nm;  $p=0.005$ ). Quadriceps endurance improved markedly with 34% after training (Figure 1D; from 136±10 to 181±18 s;  $p=0.001$ ).

Leg circumference did not change after training (from 43±1 to 44±1 cm). No increase in CSA was found in both type I (from 4224±363 to 4877±325  $\mu\text{m}^2$ ) and type II (from 3676±347 to 4235±462  $\mu\text{m}^2$ ) muscle fibers (Figure 2A). Fiber type distribution did not change after training (Figure 2B; Type I: from 35±3 to 38±3%; Type II: from 65±3 to 62±3%)

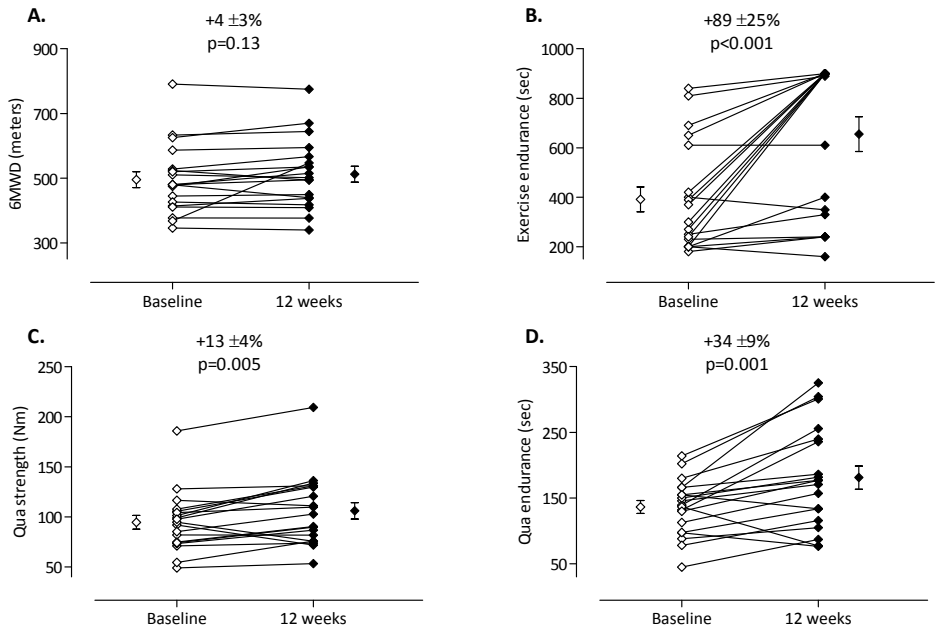
However, training increased the number of capillaries per myocyte by 30% (Figure 3; from 1.36±0.10 to 1.78±0.13;  $p<0.001$ ). SDH absorbance increased with 39% in type I (slow) muscle fibers (Figure 4; from 0.161±0.011 to 0.216±0.020;  $p<0.001$ ) whereas it increased with 30% in type II (fast) muscle fibers (from 0.105±0.009 to 0.133±0.010;  $p=0.05$ ).

The change in SDH absorbance of the type I (slow) muscle fibers and the change in the number of capillaries were highly associated to the improvements of quadriceps endurance ( $R^2=0.73$ ;  $p<0.001$ ).



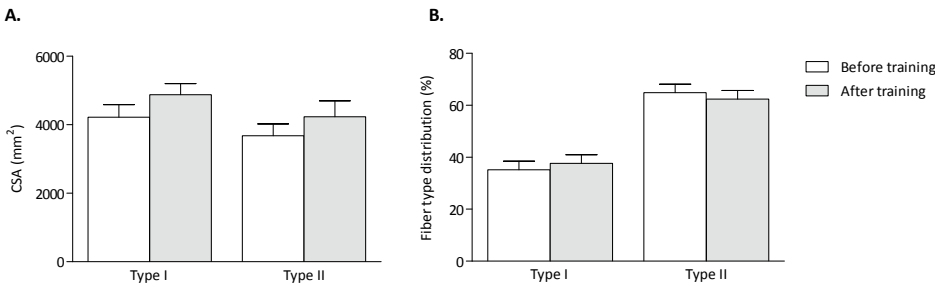
**Figure 3.1** Functional effects of exercise training

Effect of a 12 week exercise training program on six minute walk distance (A), exercise endurance (B), quadriceps strength (C) and quadriceps endurance (D). Data presented as mean  $\pm$  SEM. Open diamonds: baseline values, closed diamonds: values after training of individual patients.



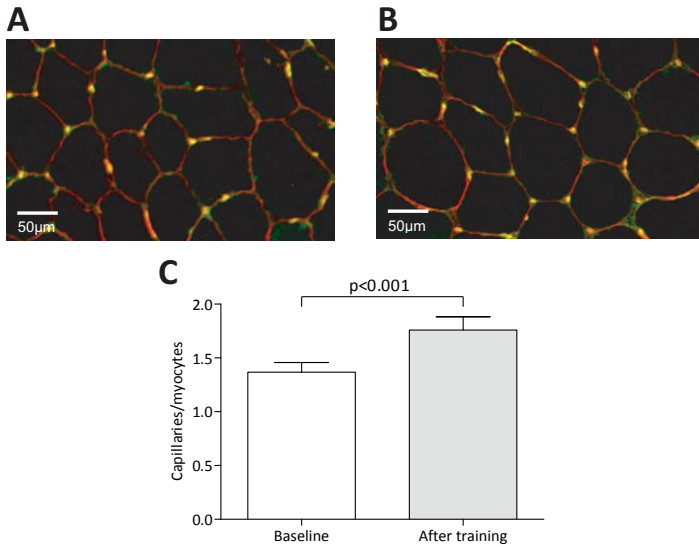
**Figure 3.2** No change in cross sectional area and fiber type distribution after training

Cross sectional area (CSA) and fiber type distribution after 12 weeks of training in both type I (slow) as in type II (fast) muscle fibers. Data are presented as mean  $\pm$  SEM. White bars: baseline values, black bars: values after training.

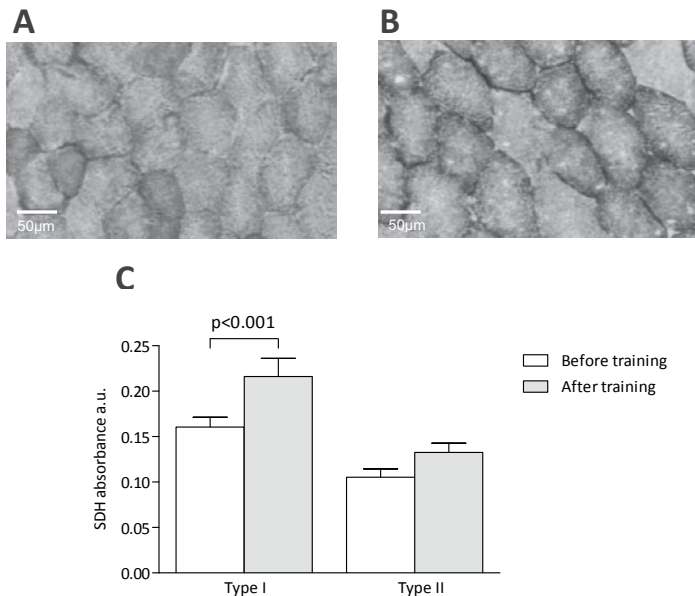


**Figure 3.3** Training increased quadriceps capillarization

Typical examples of quadriceps capillarization in one patient before (A) and after (B) training (100x magnification, red=cell membrane of the quadriceps myocytes, yellow=capillaries). Number of capillaries per quadriceps myocyte at baseline and after training (C). Data are presented as mean $\pm$ SEM.

**Figure 3.4** Training increased oxidative enzyme capacity of type I fibers

Typical examples of oxidative enzyme activity in one patient before (A) and after (B) training (dark cells indicates high oxidative enzyme activity, light cells indicates low oxidative enzyme activity). Training increased oxidative enzyme activity especially in the type I (slow) muscle fibers (C). Data are presented as mean $\pm$ SEM; White bars: baseline values, black bars: values after training. Abbreviations: SDHabsorbance: absorbance due to succinate dehydrogenase activity.



## DISCUSSION

To the best of our knowledge, this is the first study that evaluates the effect of exercise training on quadriceps function and morphology in iPAH patients. We have demonstrated that a 12-week outpatient training protocol increases endurance capacity, without an improvement of maximal capacity. The same phenomenon was seen on quadriceps function: a large improvement in quadriceps endurance was found, and a small (but significant) increase in quadriceps strength. Histological analyses revealed improved aerobic capacity by increased quadriceps capillarization and oxidative enzyme activity, without hypertrophy or fiber type switch. Finally, these morphological changes were strongly correlated with an improvement in quadriceps endurance.

### Training improved endurance capacity

In the first clinical trial by Mereles *et al*, investigating the effect of training in pulmonary hypertension, a major improvement was found in 6MWD, which was even greater than any medical intervention had achieved previously.<sup>4</sup> They also reported improvements in both maximal as well as submaximal exercise capacity. However, our study shows improvement *only* in endurance capacity. This can be explained by differences in training modalities and study population. Firstly, our training schedule consisted of cycle and quadriceps training, whereas the training schedule of Mereles *et al*.<sup>4</sup> consisted of cycling and walking training. Furthermore, our training protocol was mainly focused on improving endurance capacity, which might explain the absence of improved maximal capacity. Secondly, baseline 6MWD of our patient population were slightly higher ( $496 \pm 108$  m in our study vs.  $439 \pm 82$  m in the study of Mereles *et al*.<sup>4</sup>) which may imply that our patients had a smaller window of improvement in 6MWD. In addition to Mereles *et al*.<sup>4</sup> we were able to confirm the improvement of endurance capacity with an endurance exercise test, where we found an improvement in endurance time of 89%. Moreover, we observed the same phenomenon on quadriceps function: quadriceps strength was only modestly improved, whereas quadriceps endurance improved significantly by 34%.

Recently, Boutet *et al* presented preliminary results from their 12-week outpatient rehabilitation program in patients with iPAH.<sup>29</sup> Interestingly, they also only found improvements in endurance but not in maximal capacity, which is in line with our findings.

### Improved quadriceps endurance is associated with increased aerobic capacity

Quadriceps dysfunction is often observed in several chronic diseases such as COPD and chronic heart failure. More recently, several reports suggested muscle dysfunction in patients with pulmonary arterial hypertension.<sup>14-16</sup> Mechanisms are still unclear, but it can be speculated that inactivity of the skeletal muscles, together with a decreased cardiac output leading

to a reduced oxygen transport to the skeletal muscles, trigger morphological changes, such as muscle atrophy, fiber type switching, and reduced aerobic capacity.<sup>18</sup>

In patients with COPD or heart failure, prolonged exercise training is an effective tool to reverse changes in mitochondria, key metabolic enzymes, capillarization and - to a lesser extent - changes in fiber type composition.<sup>13</sup> These findings are in line with our findings of improved oxidative enzyme activity and number of capillaries. However, we did not find changes in CSA or fiber type distribution, which might be a consequence of the relative short exercise period at a low intensity.<sup>12</sup>

### Limitations

To reduce the burden of the present study for the patients, we limited the number of tests and the invasiveness of the measurements. We were therefore not able to assess the effect of training on quadriceps muscle function with electrical stimulation. A potential bias due to differences in motivation before and after training, may have overestimated the observed improvements in quadriceps function. However, as we found a strong association between quadriceps function and biopsy data, motivation differences may have had only a minor effect on outcome. Moreover, to limit the number of measurements, we did not measure quadriceps muscle mass by magnetic resonance imaging. Based on the measurements of leg circumference and CSA of the individual muscle fibers, changes in muscle mass are unlikely. To decrease the invasiveness of a muscle biopsy, we used a microbiopsy technique instead of the Bergström method.<sup>30,31</sup> As a consequence, protein analyses were not performed. However, we used well-validated histological techniques. For instance, capillary density was semi-automatically quantified with a CD31-antibody, which is a standard method when investigating angiogenesis.<sup>9,32</sup>

### Clinical relevance

This study confirms that iPAH is not only associated with compromised cardiopulmonary function, but also with impaired skeletal muscle function. We found that skeletal muscle dysfunction could partially be reversed by exercise training in iPAH. Moreover, our training program improved quadriceps endurance, more than quadriceps strength, even though both aspects were implemented in the training protocol. This implies that future training protocols should focus on enhancing endurance capacity rather than maximal capacity.

Although, we have found an overall beneficial effect of exercise training we cannot yet generally recommend exercise training for all PAH-patients. For instance, we were not able to rule out repercussions on cardiac function and hemodynamics, although after training NT-proBNP levels remained unaltered. Moreover, not all patients seemed to benefit from the exercise training therapy (Figure 1B). However, the relative small number of patients did not allow us to discriminate responders from nonresponders at baseline. Future studies and clini-

cal trials should assess the effects of training on right ventricular remodeling and function, and determine factors that can predict which patients can benefit most from exercise training.

#### Conclusion

Exercise training improves endurance and quadriceps muscle function, which is also reflected by structural changes of the quadriceps muscle. Our present study supports the potential role of exercise training as an adjunct therapy in stable iPAH patients.

#### Acknowledgments

The present authors would like to thank all patients who participated in the current study and the physiotherapists who supervised the training sessions.

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